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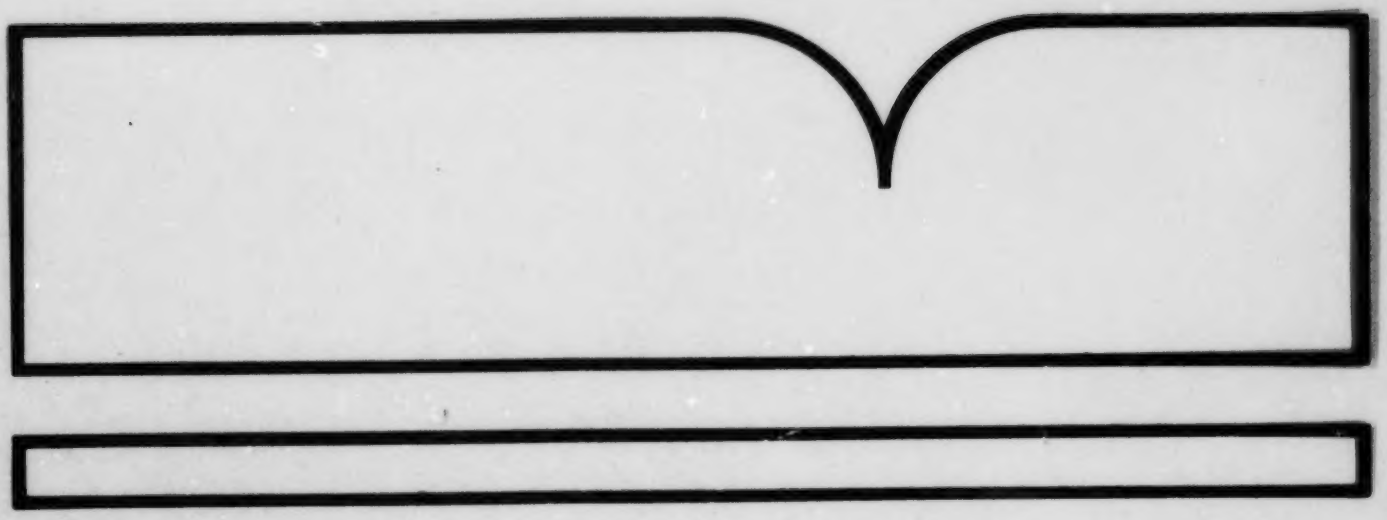

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EVALUATION OF WHO-IPCS CHEMICALS BY THE C3H10T1/2CL8 MORPHOLOGICAL TRANSFORMATION BIOASSAY

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16. ABSTRACT

In our laboratory, we have utilized C3H10T1/2 cells to study the effects of environmental chemicals on mammalian cells. During the course of these studies, we bioassayed a wide variety of chemicals including polycyclic aromatic hydrocarbons, alkylating agents, aromatic amines, azodyes, solvents, pesticides, mycotoxins, carbamates, and nitroaromatics. The results of these studies are summarized in Table 1. In the present study, we evaluated the effects of hexamethylphosphoramide (HMPA). o-toluidine, benzene, safrole, and caprolactam on oncogenic transformation of C3H10T1/2 cells. The original protocol of Reznikoff et al. (1973a) was used with minor variation.

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INTRODUCTION

Oncogenic transformation systems are valuable tools in the study of the effects of chemicals on mammalian cells. A review of these systems by the late Charles Heidelberger and his colleagues has recently been published (Heidelberger et al., 1983). The C3H10T1/2CL8 (C3H10T1/2) mouse embryo fibroblast system developed by Reznikoff et al. (1973a, b) is one of the major transformation systems using continuous (permanent) cell lines. This system has been used in the study of metabolism (Nesnow and Heidelberger, 1976; Nesnow et al., 1981); DNA binding (Amstad et al., 1983; Theall et al., 1982); cocarcinogenesis (Nesnow et al., 1981); initiation (Mordan et al., 1982, 1983); promotion (Frazelle et al., 1983; Lillehaug and Djurhuss, 1982; Mondal et al., 1976); mutation (Landolph and Heidelberger, 1979); and cytogenetics (Gehly et al., 1982).

Many variations exist on the original protocol for the C3H10T1/2 system. Treating cells at particular phases of the cell cycle produces increased cell transformation responses with direct alkylating agents (Bertram and Heidelberger, 1974; Grisham et al., 1980); reseeding treated cells produces similar results (Evans et al., 1981). In general, increases in the cells' sensitivity to chemicals are achieved by longer treatment times (Oshiro and Balwierz, 1982) and by treating colonies of cells (Nesnow et al., 1982). Short treatment times are useful when testing effects of several polycyclic aromatic hydrocarbons (Sarrif et al., 1981) while preinduction of the endogenous mixed-function oxidases increases responses with these compounds (Nesnow et al., 1981). Exogenous

metabolic activation of these cells has been reported (Benedict et al., 1978; Poole and McGregor, 1982), but it has not become a standardized technique.

In our laboratory, we have utilized C3H10T1/2 cells to study the effects of environmental chemicals on mammalian cells. During the course of these studies, we bioassayed a wide variety of chemicals including polycyclic aromatic hydrocarbons, alkylating agents, aromatic amines, azo dyes, solvents, pesticides, mycotoxins, carbamates, and nitroaromatics. The results of these studies are summarized in Table 1.

In the present study, we evaluated the effects of hexamethylphosphoramide (HMPA), o-toluidine, benzene, safrole, and caprolactam on oncogenic transformation of C3H10T1/2 cells. The original protocol of Reznikoff et al. (1973a) was used with minor variation.

MATERIALS AND METHODS

Cells and Culture Conditions

The mouse embryo fibroblast cell line C3H10T1/2CL8, derived by Reznikoff et al. (1973b), was used in these experiments. Cell cultures were incubated in humidified incubators (Hotpack Co., Philadelphia, PA) at 37°C in an atmosphere of 5% CO_2 in air. All cultures were grown in Eagle's basal medium with Earle's salts and L-glutamine supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY). Cell cultures were routinely checked for Mycoplasma infection and were found to be Mycoplasma free.

Oncogenic Transformation Assay

C3H10T1/2 cells were seeded into 60-mm Petri dishes (1000 cells in 5 ml of medium per dish, 24 dishes per experimental point). After 24 h, dishes were treated with various concentrations of safrole or caprolactam in 50 μl of dimethyl sulfoxide (DMSO) for 24 h according to the procedure of Reznikoff et al. (1973a). Dishes were unsealed during the 24-h treatment period.

For the treatment of volatiles, C3H10T1/2 cells were seeded into 25-cm^2 flasks (1000 cells in 5 ml of medium per flask, 20 flasks per experimental point). After 24 h, flasks were treated with various concentrations of HMPA, o-toluidine, or benzene in 50 μl of DMSO (Reznikoff et al., 1973a). Flasks were sealed during the 24-h treatment period.

After the 24-h treatment period, the medium was removed from the dishes or flasks and the cells received fresh complete medium containing 25 μg/ml of Garamycin sulfate (Schering Corp., Kenilworth, NJ). The medium was changed weekly until the cells reached confluency. After confluency was reached, the fetal calf serum concentration was reduced to 5% and weekly medium changes were continued.

At the end of 6 wk, the cells were washed with a 0.85% NaCl solution, fixed with methanol, stained with Giemsa, and scored for oncogenic transformation by the following criteria: Type I, Type II, and Type III foci.

Positive control cultures were treated with benzo(a)pyrene (B(a)P) (1 μg/ml); negative cultures were treated with DMSO (1%). The protocol was the same as that used for experimental cultures. Cytotoxicity assays were run concurrently with transformation assays. The protocol was the same as that used for experimental cultures with the following exceptions: dishes or flasks were seeded with 200 cells, there were 6 dishes or flasks per chemical concentration tested, and both were stained 10 - 12 d after seeding.

Statistical Analysis

In general, the data did not indicate dose-related responses for the 5 chemicals tested. For this reason, Fisher's Exact Test was used as an alternative to the multiple logistic model previously used in similar experiments (Nesnow et al., 1981).

RESULTS

The results of the bioassays of HMPA, o-toluidine, benzene, safrole, and caprolactam are shown in Tables 2 - 6. Benzo(a)pyrene was used to treat positive control cultures in each experiment; it transformed cells in 46 - 90% of these cultures. In total, we observed foci in 52 of the 84 B(a)P-treated cultures (62%). We observed 43 Type II and 38 Type III foci in these cultures for an average of 0.85 foci per dish/flask. Spontaneous transformation was not detected in any of the 84 dishes/flasks containing DMSO-treated negative control cultures; this represents a spontaneous transformation frequency of <0.005%. Our laboratory's historical spontaneous transformation frequency is 0.0001%.

The chemicals we tested are of low molecular weight. To remain within reasonable physiological concentrations, 1000 μg/ml was selected as the highest concentration tested in the absence of cytotoxicity. Therefore, HMPA, benzene, and caprolactam were not tested at concentrations greater than 1000 μg/ml.

Marginally statistically significant levels of transformation ($p = 0.12$) were observed with HMPA and benzene tested at 1000 μg/ml (Tables 2, 4); tests with o-toluidine and safrole (Tables 3, 5) produced significant levels of transformation at 500 μg/ml ($p = 0.02$) and 100 μg/ml ($p = 0.06$), respectively. Caprolactam was considered inactive.

DISCUSSION

In this study, tests on benzene and HMPA produced marginal results; o-toluidine and safrole induced significant transformation. Caprolactam was inactive. Examination of these chemicals at more closely spaced dose levels might have produced additional evidence of dose-related responses (especially if response curves were steep).

C3H10T1/2 cells contain microsomal cytochrome P-450 and epoxide hydrolase enzymes required for the metabolic activation of many chemical carcinogens (Nesnow et al., 1981). Recent results in our laboratory indicate that the active conjugating systems UDPGA-glucuronyl transferase and PAPS-sulfotransferase are present in these cells. It is, therefore, not surprising that C3H10T1/2 cells can activate safrole and o-toluidine into forms which then are able to transform them. Additional investigations are needed to confirm the observed oncogenic transformation of C3H10T1/2 cells by these two chemicals and to probe the mechanisms of metabolic activation.

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This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

TABLE 1. SUMMARY OF CHEMICALS BIOASSAYED IN C3H10T1/2CL8 CELLS[a]

Chemical	Result[b]	Minimum Detectable Concentration or Highest Concentration Tested (μg/ml)	Carcinogenicity[c]
Polycyclic Aromatic Hydrocarbons			
Benzo(a)pyrene	+	0.1	C
Cyclopenta(cd)pyrene	+	0.3	C
7,12-Dimethylbenz(a)anthracene	+	0.3	C
3-Methylcholanthrene	+	0.3	C
Benz(a)anthracene	-	6.8	C
5,6-Benzoflavone	-	9.0	?
7,8-Benzoflavone	-	6.8	?
1,2-Dihydronaphthalene	-	6.5	?
1,2-Naphthoquinone	-	0.47	?
Phenanthrene-9,10-quinone	-	0.62	?
Styrene	-	5.0	C
Alkylating Agents			
N-Acetoxy-2-fluorenylacetamide	+	1.0	C
Cyclopenta(cd)pyrene-3,4-oxide	+	0.3	?
Ethylnitrosourea	+	150	C
N-Methyl-N´-nitro-N-nitrosoguanidine	+	0.3	C
Propane sultone	+	40	C

TABLE 1. (CONTINUED)

Chemical	Result[b]	Minimum Detectable Concentration or Highest Concentration Tested (μg/ml)	Carcinogenicity[c]
β-Propiolactone	+	0.1	C
Cyclohexene oxide	-	5.0	?
Styrene oxide	-	6.0	C
Aromatic Amines/Azo Dyes			
4-Dimethylaminoazobenzene	-	22.5	C
2-Fluorenylacetamide	-	10	C
Solvents			
Acetone	-	1%	?
Dimethyl sulfoxide	-	1%	?
Ethanol	-	9.3	?
Other Chemicals			
Aflatoxin B_1	+	1.0	C
4-Nitroquinoline-N-oxide	+	0.003	C
Trichlorfon	+	3.0	?
Toxaphene	+/-	12.4	C
Acephate	-	30	?
Aroclor-1254	-	9.8	?

TABLE 1. (CONTINUED)

Chemical	Result[b]	Minimum Detectable Concentration or Highest Concentration Tested (μg/ml)	Carcinogenicity[c]
4-Cnloro-o-toluidine	-	106	C
Chlordimeform	-	100	?
Cyclohexene	-	4.1	?
p,p´-DDT	-	11	C
2,7-Dinitrofluoren-9-one	-	30	?
Hexachlorobenzene	-	14	C
Nickel sulfate	-	100	?
Phenobarbital	-	25.4	?
Pregnenolone-16-α-carbonitrile	-	34	?
Trifluralin	-	1.0	?
2,4,7-Trinitrofluoren-9-one	-	1.0	?
Urethane	-	1000	C
Vinyl carbamate	-	1000	C

[a]Bioassays performed in the author´s laboratory.

[b]+, a statistically significant dose-related response of Type II and Type III foci. -, no response at the highest concentration tested.

[c]C, known carcinogen.

TABLE 2. EFFECT OF HMPA ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H10T1/2 CELLS[a]

Conc. (μg/ml)	Survival (% Control)	No. Flasks Scored	No. Type I Foci	No. Type II Foci	No. Type III Foci	% Flasks with Type II & Type III Foci
0	100	20	0	0	0	0
250	117	20	1	0	0	0
500	118	20	0	0	0	0
750	132	20	0	1	0	5
1000	138	20	0	1	2	15[b]
B(a)P						
1	32	20	0	4	10	55

[a]C3H10T1/2 cells exposed to chemical in sealed flasks.

[b]p = 0.12, Fisher's Exact Test.

TABLE 3. EFFECT OF o-TOLUIDINE ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H10T1/2 CELLS[a]

Conc. (μg/ml)	Survival (% Control)	No. Flasks Scored	No. Type I Foci	No. Type II Foci	No. Type III Foci	% Flasks with Type II & Type III Foci
0	100	20	0	0	0	0
250	108	20	0	0	0	0
500	86	20	0	4	1	25[b]
750	71	20	0	1	2	10
1000	52	20	0	1	0	5
B(a)P						
1	59	20	3	22	12	90

[a]C3H10T1/2 cells exposed to chemical in sealed flasks.

[b]p = 0.02, Fisher's Exact Test.

TABLE 4. EFFECT OF BENZENE ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H10T1/2 CELLS[a]

Conc. (μg/ml)	Survival (% Control)	No. Flasks Scored	No. Type I Foci	No. Type II Foci	No. Type III Foci	% Flasks with Type II & Type III Foci
0	100	20	0	0	0	0
250	103	20	0	1	0	5
500	92	20	0	0	0	0
750	116	20	0	1	0	5
1000	101	20	0	2	1	15[b]
B(a)P						
1	11	20	1	7	12	60

[a]C3H10T1/2 cells exposed to chemical in sealed flasks.

[b]p = 0.12, Fisher's Exact Test.

TABLE 5. EFFECT OF SAFROLE ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H10T1/2 CELLS[a]

Conc. (μg/ml)	Survival (% Control)	No. Dishes Scored	No. Type I Foci	No. Type II Foci	No. Type III Foci	% Dishes with Type II & Type III Foci
0	100	24	0	0	0	0
10	90	24	0	0	0	0
25	54	24	0	0	0	0
50	40	24	0	1	1	8
100	35	24	1	6	0	17[b]
200	0	24	1	1	0	4
B(a)P						
1	31	24	1	10	4	46

[a]C3H10T1/2 cells exposed to chemical in open dishes.

[b]p = 0.06, Fisher's Exact Test.

TABLE 6. EFFECT OF CAPROLACTAM ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H10T1/2 CELLS[a]

Conc. (μg/ml)	Survival (% Control)	No. Dishes Scored	No. Type I Foci	No. Type II Foci	No. Type III Foci	% Dishes with Type II & Type III Foci
0	100	24	0	0	0	0
200	102	24	0	0	0	0
400	107	24	0	0	0	0
600	101	24	0	0	0	0
800	90	24	0	0	0	0
1000	93	24	0	1	0	4
B(a)P						
1	31	24	1	10	4	46

[a]C3H10T1/2 cells exposed to chemical in open dishes.

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